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AWARD NUMBER: W81XWH-05-1-0270

TITLE: Gene Therapy for Osteolytic Breast Cancer Bone Metastasis

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REPORT DATE: June 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
1 June 2009	Final	1 Jun 2005 – 31 May 2009

4. TITLE AND SUBTITLE

Gene Therapy for Osteolytic Breast Cancer Bone Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0270

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Alabama at Birmingham
Birmingham, AL 35291

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. We sought to achieve sustained effects of OPG combining cell therapy and gene therapy approaches. The aims were to determine therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, and to determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis. Through this funding, we produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of $\alpha 4\beta 1$ integrin. Combination of chemotherapy with OPG therapy indicated that

15. SUBJECT TERMS
Osteolysis, osteoprotegerin, mesenchymal stem cells, gene therapy, adeno-associated virus

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	14	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Title of the Grant: Gene therapy for osteolytic breast cancer bone metastasis
Award number: W81XWH-05-1-0270
Principal Investigator: Selvarangan Ponnazhagan, Ph.D.
Final Report: June 01, 2005 – May 31, 2009

INTRODUCTION

Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of cancer cells and the bone microenvironment results in the upregulation of factors promoting osteoclastogenesis and osteolytic bone destruction. Thus, osteolysis and tumor cell accumulation can be inhibited by interrupting one or more of the steps involved in the cycle. The major treatment to reduce the burden of bone metastasis in breast cancer patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, while improving the formulations of bisphosphonate compounds, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial.

A better understanding of the molecular events in breast cancer osteolytic bone destruction indicates that the receptor activator of nuclear factor κ B ligand (RANKL), produced by osteoblasts, activated T cells and marrow stromal cells stimulates the recruitment, differentiation, and activation of osteoclasts by binding to RANK. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. To achieve sustained effects of OPG, gene therapy is more powerful than pharmacological therapies. Since the process of bone metastasis in breast cancer is a secondary event that occurs in late-stage disease or during recurrence, genetic therapies aimed at controlling this process should be both sustained and localized. Thus, for sustained expression of therapeutic levels of OPG, a vector capable of stable expression of the transgene without vector-associated toxicity and immunity is ideal. The adeno-associated virus vectors (AAV) are more promising to this end. With recombinant AAV vectors, it is possible to obtain significant therapeutic advantage by either systemic or bone-targeted transduction and can be combined with bisphosphonate treatment for synergistic effects.

The proposed specific aims of the project are:

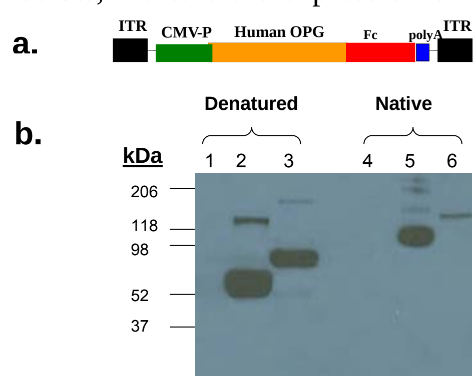
1) To determine the therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, *and*

2) To determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis.

BODY

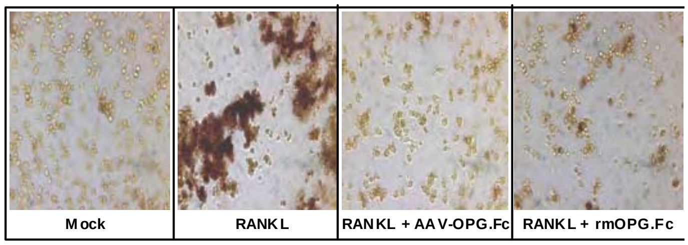
Year 1:

Production of adeno-associated viral vectors encoding human OPG and analysis of expression as a soluble factor. We constructed recombinant adeno-associated virus vectors (rAAV) encoding osteoprotegerin (OPG), either as a fusion protein to the human immunoglobulin (Fc) or without Fc. The constructs were tested initially for the expression and extracellular secretion of OPG in RAW (a murine macrophage cell line) cell cultures. Results, shown in figure below, indicate the expression of OPG from rAAV transduced cells.



Recombinant AAV encoding the human OPG.Fc (a) and expression of the OPG.Fc from RAW cell supernatant (b). RAW cells were mock-transduced or transduced with rAAV-OPG.Fc construct and the supernatants were analyzed by Western blot using a monoclonal antibody for human OPG in either denatured gel or non-denatured gel. Lane assignments: Lane 1&4: Mock; 2: s-hOPG-Fc; 3: hOPG-Fc; 4: Mock 293; 5: s-hOPG-Fc; 6: hOPG-Fc

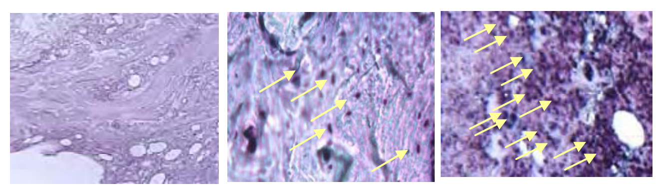
Transduction of rAAV-OPG.Fc inhibits osteoclast differentiation *in vitro*. The biological activity of rAAV produced OPG was determined in osteoclast forming assay using RAW cells. Briefly, 10^5 RAW cells were plated in 24-well tissue culture plates and grown in medium containing 10% FBS, 20 ng/ml M-CSF, and 50 ng/ml RANKL in the presence or absence of conditioned medium from 293 cells transduced with rAAV-OPG.Fc. The growth medium plus additives were changed every alternate day. After five days of culture, the cells were fixed and stained for tartrate-resistant alkaline phosphatase (TRAP), a marker for multinucleated osteoclasts. Results, shown in figure below, demonstrate that rAAV produced OPG is biologically active and effectively inhibits osteoclastogenesis.



TRAP assay of RAW cells following rAAV-OPG.Fc transduction.

Recently, we established that mesenchymal stem cells (MSC), to be used in the proposal as cell therapy and gene therapy vehicle, can be efficiently transduced by AAV (1). In order to increase their efficacy in bone-specific homing by ex vivo method, we adapted a strategy to transiently express α4β1 integrin on MSC cell surface. By this modification, we were able to successfully increase bone specific homing and retention of MSC upon in vivo transfer. This is shown in Figure below.

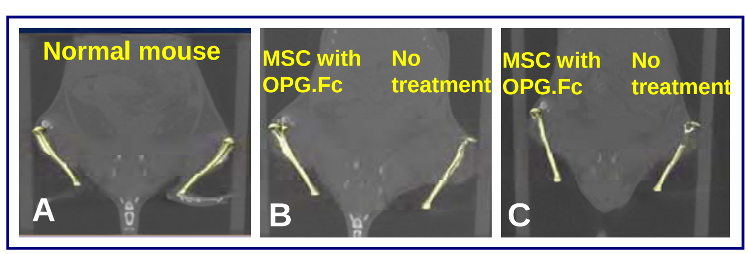
***In situ* hybridization analysis of bone marrow stromal cells for the identification of donor MSC repopulation.** Two weeks after sham-transplantation (Normal), or transplantation with mock-tranfected (MSC only) or transfected with plasmid encoding α4 integrin (MSC with α4 integrin) to mouse stem cells from male mice, animals were sacrificed for analysis. Femur bones were decalcified and sectioned to 5 μm thickness. The sections were deparaffinized and rehydrated through alcohol gradation series. Following denaturation at 85o C in hybridization buffer, the sections were probed using DIG-labeled Y-chromosome specific DNA probe. The slides were gently counterstained with eosin.



Normal **MSC only** **MSC with α4 integrin**

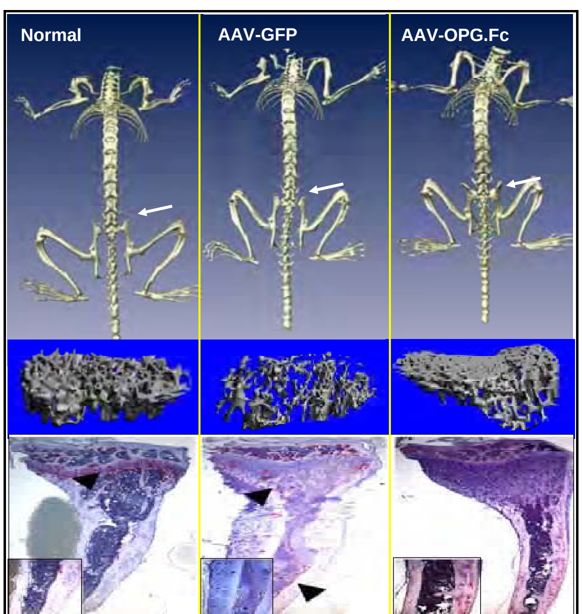
To test the efficiency of OPG-expressing MSC in protecting osteolytic lesions due to cancer bone metastasis, encountered commonly in breast cancer patients, we transfected OPG expression vector in mouse MSC and transplanted them to tibial bones of nude mice harboring osteolytic cancer cell line, PC-3. These cells were stably transfected with luciferase gene, hence, allowed non-invasive imaging of the cancer cell growth. Micro-CT analysis of the bone following the therapy indicated remarkable retention of bone architecture after the OPG-expressing MSC were therapeutically implanted.

Radiographic images of mice tibia following treatment with MSC producing OPG.Fc. Approximately 10^5 osteolytic bone metastatic cancer cell line PC-3 were implanted in mouse tibia (B & C). Seven days after tumor cell implantation, MSC producing OPG.Fc was injected in one side and the other side left untreated. Picture shown in panel A is from a normal mouse without any tumor or MSC.



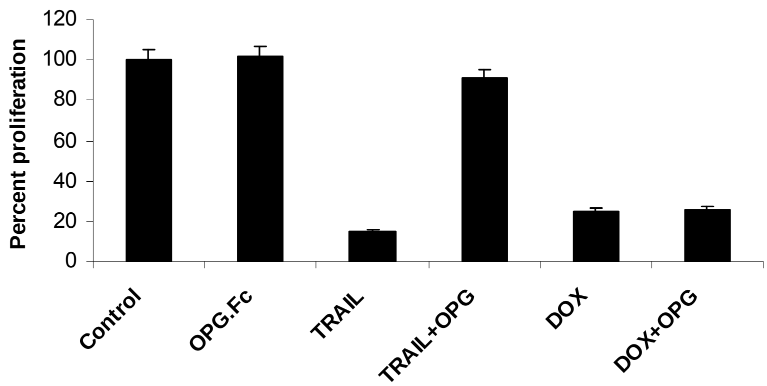
Year 2:

Recombinant AAV-mediated, systemically stable expression of osteoprogering inhibits osteolytic bone damage. The purpose of the present study was to determine the effect of sustained level of OPG.Fc in a therapeutic model of osteolytic breast cancer. 6 weeks old female athymic nude mice (n=24) were injected intra-cardiac 2 X 10^5 MDA-MB-435 breast cancer cells expressing firefly luciferase. 3 mice served as normal age matched control and received no tumor cells or treatment. Widespread skeletal metastases were confirmed 7 days after the intra-cardiac injection after bioluminescence imaging. 16 out of 24 mice showed clear signs of bone metastasis. At this point 8 out of 16 positive mice were administered intra-muscular delivery of 3 X 10^{11} virus particles of AAV-OPG.Fc and rest of the mice received 3 X 10^{11} particles of AAV-GFP. Therapeutic benefits were determined 4 weeks after the delivery of the virus particles. Bioluminescence imaging showed significant reduction in tumor growth in the OPG.Fc treated mice compared to GFP treated mice. No significant difference was observed in bioluminescence from any extra-osseous metastasis between OPG.Fc and GFP treated mice. Micro-computerized tomography (μCT) analysis indicated significant decline in bone to tissue volume ratio and trabecular bone density in the tumor challenged untreated or GFP treated animals. OPG.Fc treatment restored the bone to tissue volume ratio and significantly enhanced the trabecular bone density. Ki-67 immunstaining showed presence of proliferating tumor cells in the tibia of both OPG.Fc treated and GFP treated animals. PARP-p85 immunostaining was detected only in the tumor cells which are trapped inside the trabeculae of the newly formed cancellous or trabecular bone in the metaphyses and not in the tumor cells those were present in the diaphyses suggesting indirect killing of tumor cells by OPG.Fc. A significant loss in body weight was observed in both OPG.Fc treated and GFP treated mice which suggest inability of OPG.Fc to influence any soft tissue metastasis. Moreover, OPG.Fc treatment resulted into overproduction of bone, which may require introduction of regulated gene expression system. We suggest that OPG.Fc in combination with chemotherapy may prove useful for the management of osteolytic bone metastasis. Results of these studies are shown in figure below.

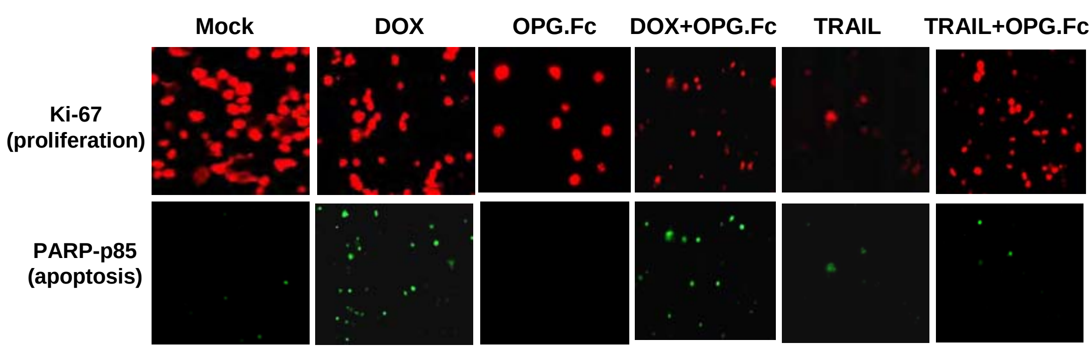


Micro-CT analysis and trichrome staining of bone following rAAV-OPG.Fc transduced MSC therapy for tumor- induced bone loss. Micro-CT analysis of entire skeleton is provided in top. Arrows point to bone restoration in pelvic region following therapy. ***Architecture of trabecular bone*** (middle panel) was determined by micro-CT after sacrificing mice. Trichrome staining showing decreased osteoclast activity following rAAV-OPG.Fc therapy is shown in the bottom panel.

Doxorubicin induces cancer cell apoptosis independently of TRAIL blocking by OPG. Since TRAIL binding property of OPG inhibits apoptosis of metastasized cancer cells in the bone marrow, we reasoned that combination of OPG therapy with chemotherapy using drugs capable of inducing cancer cell apoptosis, independently of TRAIL pathway, would provide great synergy. To determine this, we used doxorubicin, a known chemotherapeutic drug commonly given to patients with breast cancer. Human osteolytic cancer cell line line, CAG, was seeded at a concentration of $2x10^5$ cells/well of a 6-well dish and treated with 50 ng/ml TRAIL, 1 μg/ml purified OPG.Fc and 20 ng/ml doxorubicin in various combinations. The cells were harvested after 48 hours, washed in PBS and subjected to Trypan Blue staining for cell viability. Cell proliferation was determined by MTT assay and immunostaining performed in a cytospin using cell proliferation marker Ki-67 and apoptosis marker PARP-p85 fragment. Results of these studies, shown in figures below demonstrate that although in the presence of OPG, TRAIL-mediated apoptosis is significantly prevented, apoptotic effects of DOX was totally unaffected in the presence of OPG. Thus, combination of DOX with bone-targeted OPG therapy is expected to prevent osteolytic bone damage and promote tumor cell apoptosis to increase survival. Results of these studies are shown in Figure below.



Tumoricidal effects of doxorubicin is independent of TRAIL binding of OPG. Human osteolytic cancer cell line, CAG, were seeded at a concentration of 2 X 10^5 cells/well of a 6 well dish and treated with 50ng/ml TRAIL, 1 μg/ml mouse OPG .Fc and 2 0ng/ml doxorubicin (DOX) in v arious combinations i n duplicates. MTT assay for cell proliferation was performed 48 hrs later.



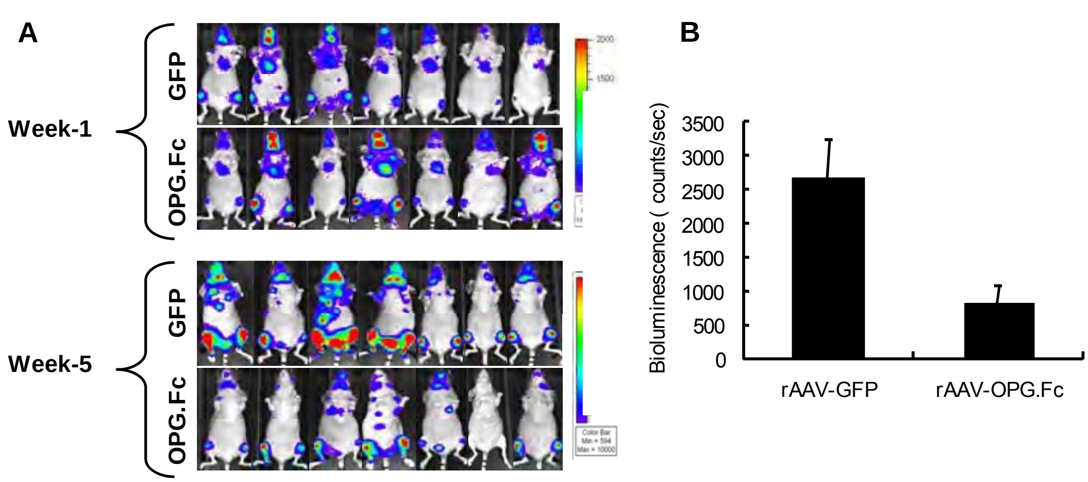
Proliferation and apoptosis of human osteolytic cancer cell line following treatments with OPG, DOX and TRAIL combinations. Human oste olytic cell line CAG was se eded at a con centration o f 2 X 10^5 cells/well of a 6 well dish and treated with 50ng/ml TRAIL, 1 μg/ml mouse OPG.Fc and 20ng/ml doxorubicin in various combinations in duplicates. Cells were harvested after 48 hours, centrifuged to pellet the cells, washed in PBS and analyzed by immunostaining for cell proliferation marker (Ki-67-Alexa fluor-594,red) and apoptosis marker (PARP-p85 fragment, Alexa Fluor-488, green).

Year 3:

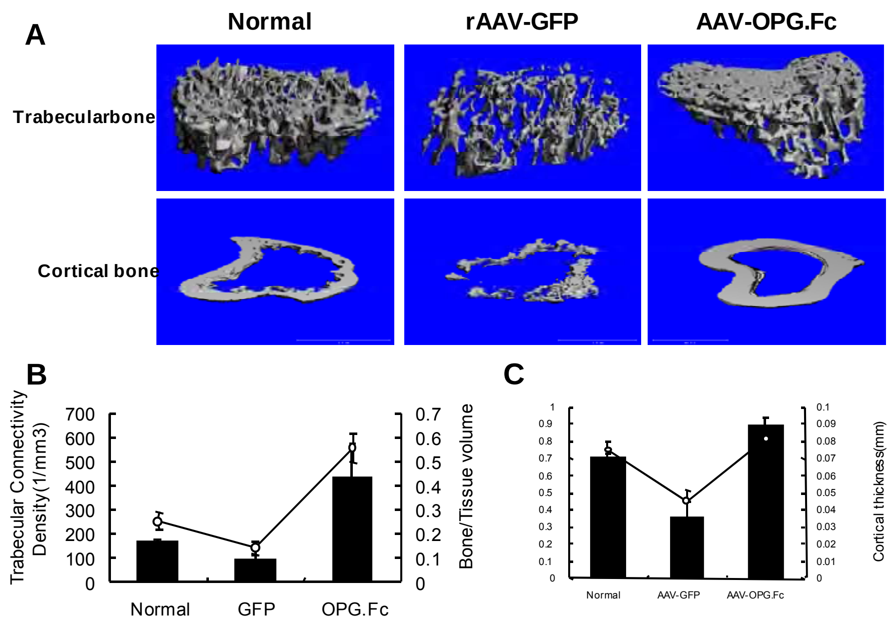
In year 3, we characterized the nature of the treatment effects both on bone and on the cancer cells. As well, we determined the toxicity-related issues of this treatment. Following is a brief account of the outcome.

We have used both MDA-MB-435 and MDA-MB-231 cells initially and found the MDA-MB-435 cells to be better in bone mtastasis. Although some controversy exists over the origin of the MDA-MB-435 cells the these cells express melanoma-associated genes. MDA-MB-435 cells were isolated from a patient reported to have breast cancer and express breast-specific or epithelial-specific markers. MDA-MB-435 cells were also induced to express breast differentiation-specific proteins and secrete milk lipids as observed in other well-established breast cancer cell lines, suggesting more a breast cancer phenotype (1). Furhter, they make osteolytic bone lesions similar to the situation in human breast cancer patients. In our study, the main focus was to develop treatment strategies for osteolytic bone damage in breast cancer. Hence, we continued to use this cell line. A paper from this work was published in Molecular Therapy *(Mol. Ther. 2008, 16:871-878)* by us, qualifying the strategy as a potential approach for osteolytic breast cancer bone metastasis.

Tumor growth following OPG.Fc therapy. MDA-MB-435 breast cancer cells expressing luciferase were injected into the left ventricle of the heart of 6 weeks old athymic nude mice. Apparent signs of skeletal metastasis were observed by bioluminescence imaging, seven days after the intra-cardiac delivery of MDA-MB-435 cells in the skull, vertebral column, femur, tibia and humerus. Some retention of the injected tumor cells was also observed in the heart. On day 8, 3 $x10^{11}$ genomic particles of rAAV-6 encoding either OPG.Fc or GFP suspended in normal saline, were injected in in the quadriceps muscle of the hind limb. Four weeks later, mice were imaged again for luciferase expression. Growth of the tumor cells was evident in both treatment groups. Comparison of bioluminescent images between the two treatment groups clearly indicated that the progression of tumor growth was significantly less in rAAV-OPG.Fc treated mice than rAAV-GFP treated mice. Bioluminescence imaging also indicated that metastasizing MDA-MB-435 cells predominantly disseminated within the skeleton as compared to other organs. However, luminescent intensity of tumor cells in the bones indicated a significant reduction (30.22%) of tumor growth in rAAV-OPG.FC treated group compared to rAAV-GFP treated mice ($p<0.02$). Results of these studies are given below.

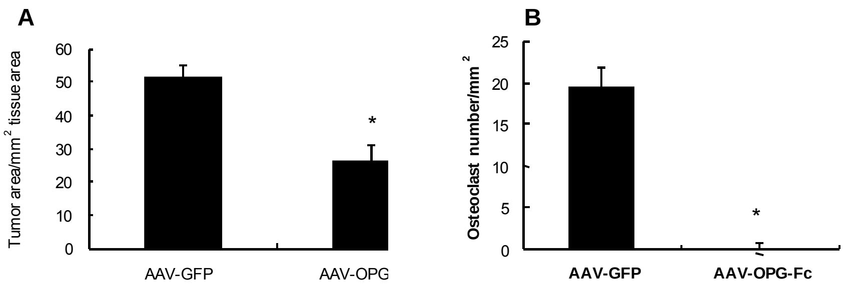


Effect of OPG.Fc therapy on bone remodeling. MDA-MB-435 breast cancer cells most frequently metastasized into the tibia compared to other parts of the skeleton. Results described are from the tibia of different experimental groups of mice. Three-dimensional μCT data indicated a significant ($p<0.001$) decline in relative trabecular bone volume and trabecular connectivity density in rAAV-GFP treated mice as compared to normal mice. However, OPG.Fc treated mice showed the highest amount of relative trabecular bone volume and trabecular connectivity density when compared to both GFP treated mice and normal mice. Micro-CT analysis of the cortical bone in the metaphysis suggested a significant ($p<0.005$) decline in bone volume and cortical thickness in GFP treated mice as compared to normal mice. Significant restoration of cortical bone was observed in the OPG.Fc treated mice ($p<0.001$). These results are shown in the figure below.

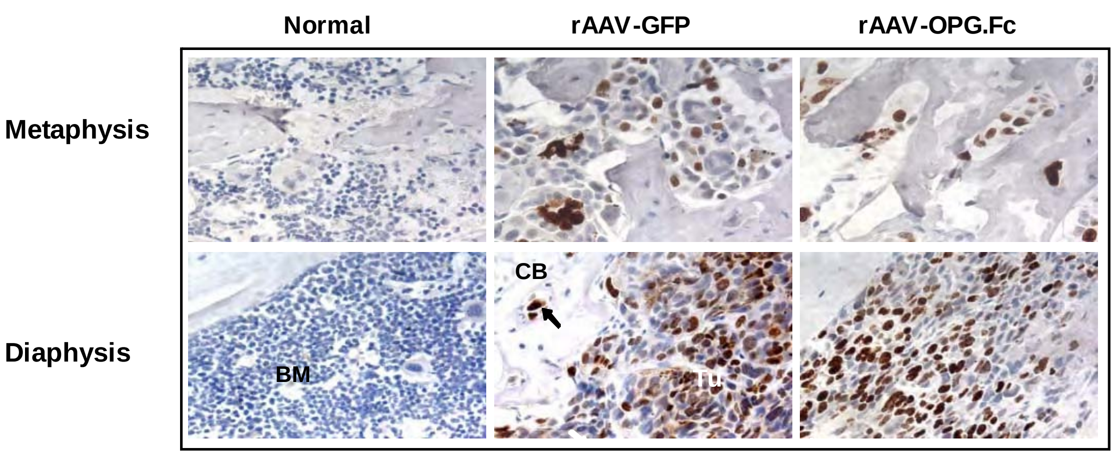


No significant difference was observed in cortical bone volume and thickness between various treatment groups when diaphysis was analyzed (data not shown). Histomorphometry of the tibia was also performed in addition to μCT with H&E and Goldner's trichrome stain. Results of this analysis strongly supported μCT observations and indicated that in untreated mice with tumor or rAAV-GFP treated mice, tumor cells replaced the bone marrow completely by 5 weeks after intra-cardiac injection of MDA-MB-435 cells. In rAAV-GFP treated mice, prominent bone damage was noticed both in the trabecular and in cortical bone areas (Figure 3A). The majority of the tumor cells were present in the metaphyseal region, which also displayed maximum bone destruction. In the diaphyses, tumor cells invaded into the calcified cortex and were found to grow in small pockets initially, eventually leading to complete destruction of the bone (Figure 3A). Despite the presence of prominent osteolysis in the GFP treated mice, thickening of the cortical bone in the diaphysis could be seen in the sections of tibia suggesting existence of an osteoblastic phenotype of this cell line. Rarely the tumor cells were seen in the epiphysis above the growth plate in both femur and tibia. OPG.Fc therapy significantly protected both the cortical and trabecular bone from osteolysis (Figure 3A). H&E and Goldner's trichrome staining of the bone sections showed a remarkable increase in trabecular bone volume in the OPG.Fc treated mice as compared to GFP treated mice. Trabecular bone volume, in fact, exceeded in the OPG.Fc treated mice than that of the age-matched normal mice. This resulted in a significant reduction ($p<0.02$) in tumor burden in OPG.Fc treated mice (Figure 3B). In the OPG.Fc treated mice, the metaphysis region of the tibia was almost devoid of any tumor cells, instead replaced by newly formed or trabecular bone.

TRAP staining was performed to determine the number of osteoclasts present following the therapy (Figure 3A). In GFP treated mice, the osteoclasts were located at the entire tumor/bone interface, including both trabecular and cortical bone surfaces. In age-matched normal mice, osteoclasts were mostly located in the trabecular bone in the metaphysis of the tibia. The size of osteoclasts in GFP treated mice was significantly larger than the osteoclasts in normal mice (Figure 3A). In normal mice, significant numbers of osteoclasts were present at the growth plate whereas virtually no osteoclast was located at the growth plate of mice with tumor. OPG.Fc therapy almost completely abolished the formation of mature osteoclasts as evidenced by the absence of any TRAP positive cells in the tibia. Computer-assisted quantitation of osteoclasts indicated a highly significant reduction in the number of osteoclasts in OPG.Fc treated animals compared to AAV-GFP treated mice ($p<0.001$; Figure 3C).

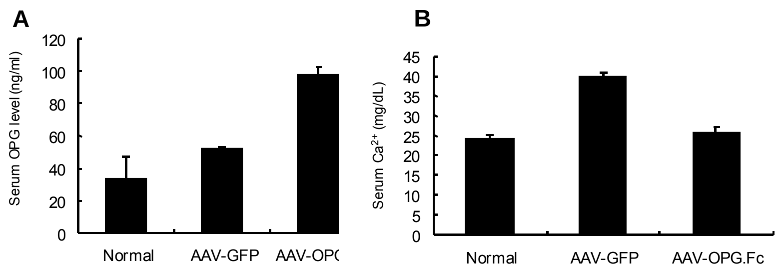


Tumor cell proliferation. Ki-67 immunostaining were performed to determine the effect of rAAV-OPG.Fc therapy on tumor cell proliferation in the bone (Shown in Figure below). Positive Ki-67 immunostaining was observed in tumor cells of both GFP treated and OPG.Fc treated mice. However, in rAAV-OPG.Fc treated mice, there was a wave of newly formed trabecular bone in the metaphyseal region of the tibia and Ki-67 immunoreactive tumor cells were mostly restricted to the diaphysis. No PARP-p85 immunostaining was observed in the tumor cells present in the tibia of rAAV-OPG.Fc treated mice, indicating OPG.Fc failed to induce apoptosis in the tumor cells (data not shown).

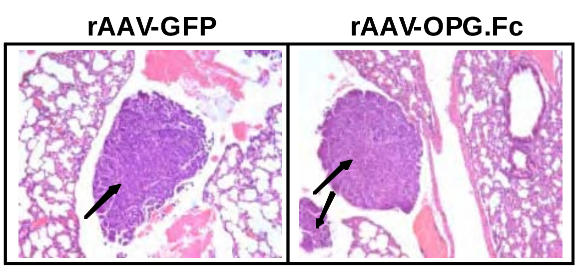


Systemic levels of OPG and ionized calcium. ELISA was performed on serum samples obtained from rAAV-OPG.Fc treated mice at the end of the experiment to determine the systemic levels of OPG.Fc (Figure 5A). Highest level of circulating OPG.Fc (110.75 ng/ml) was obtained by 1 week after intra-muscular delivery of rAAV and steadily maintained until the termination of the experiment. The ELISA kit used in this study detects the full-length human OPG, hence, a possibility exists that levels of OPG.Fc in treated mice include contributions from both mouse OPG and OPG produced by the breast cancer cells. Therefore, to determine the actual level of OPG.Fc produced by the rAAV vector we measured the circulating OPG level in the rAAV-GFP treated mice and age matched normal mice. rAAV-OPG.Fc treated mice showed significantly higher OPG level as compared to both rAAV-GFP treated and age-matched normal mice ($p<0.001$). All samples were run in a single assay and intra-assay variation was <10% while the sensitivity of the assay was found to be 3 pg/ml.

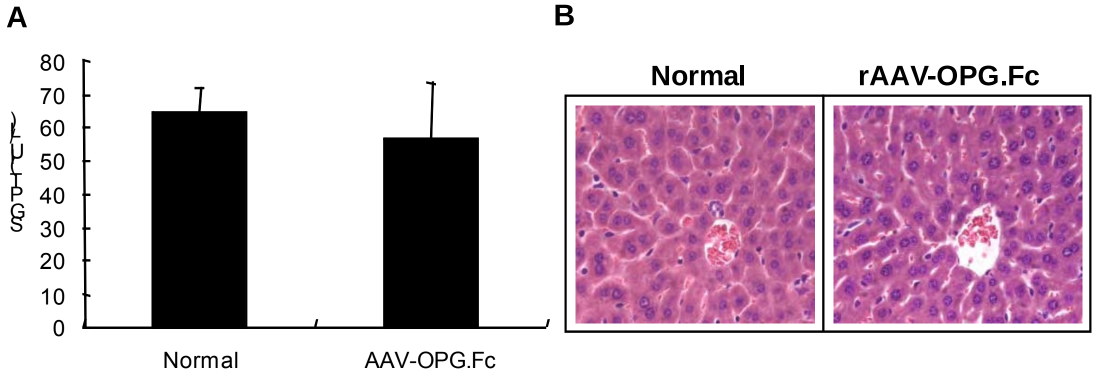
Hypercalcemia is characteristic of osteolysis due to bone metastasis of breast cancer cells. To determine the protective effect of OPG.Fc therapy on serum calcium levels, we measured the ionized serum calcium level in GFP treated and OPG.Fc treated mice as compared to normal mice. Serum calcium was higher in the GFP treated, tumor bearing mice when compared to OPG.Fc treated or normal mice ($p<0.001$), suggesting OPG.Fc therapy prevents breast cancer related hypercalcemia by preventing osteolysis (Figure below).



Effect of rAAV-OPG.Fc therapy on metastasis to other organs. Multiple organs were studied besides bone for metastasis including liver, lung, spleen, lymph nodes, heart, kidney, adrenal gland and occasionally gallbladder. Metastases were noted mainly in the myocardium of the heart, in the lungs and in the adrenal glands. However, no therapeutic advantage was observed in rAAV-OPG.Fc treated mice compared to rAAV-GFP treated mice in preventin g metastasis to non-osseous sites (Figure below). Moreover, the OPG.Fc treated mice lost 25% of their body weight similar to the GFP treated mice by 5 weeks after the inoculations of the tumor cells and were euthanized at the same time.

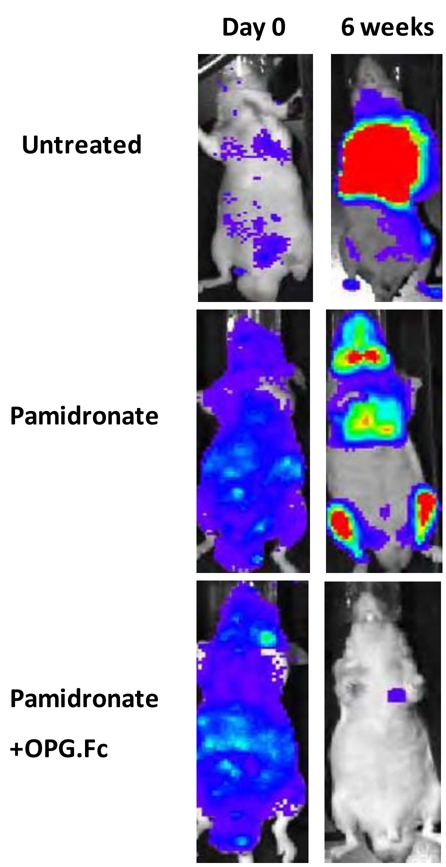


Systemic rAAV-OPG.Fc therapy does not induce hepatotoxicity. As a measure of liver toxicity, SGPT activity and liver histopathology were performed and compared to control mice, which did not receive any vector. Liver sections were stained with H&E and observed under the microscope. Normal liver morphology in both rAAV-OPG.Fc treated and control mice suggested the absence of toxicity because of systemic OPG.Fc therapy. No significant difference in SGPT levels was observed between age matched normal mice and the OPG.Fc treated group ($p>0.6$; Figure below)**.**



Year 4 (no-cost extension period):

During the no-cost extension of the grant, we conducted studies to determine the significance of OPG therapy in combination with bisphosphonate therapy. $2X10^5$ MDA-MB-435 cells expressing firefly luciferase were injected via intra-cardiac route in 6 week-old female athymic nude mice. Seven days after the inoculation of the tumor cells, the mice were administered 10mg/Kgbw pamidronate alone (ip) or alongwith $3X10^{11}$ particles of rAAV expressing human OPG.Fc (im). Tumor growth was monitored by bioluminescence imaging until 5 weeks after the initiation of treatment. Results indicated significant inhibition of tumor growth in mice treated with pamidronate in combination with rAAV-OPG.Fc compared to pamidronate alone and untreated animals. Representative image showing a significant decrease in the tumor growth and organ dissemination following the combination therapy.



KEY RESEARCH ACCOMPLISHMENTS

Year 1:

- Develped rAAV encoding human OPG, produced high-titer virus and validated the biological efficacy of the vector encoded protein in inhibiting osteoclastogenesis in vitro.
- Developed strategy to increase bone-specific homing of MSC.
- Established that MSC expressing OPG greatly reduce ostelytic effects of cancer growth in bone.

Year 2:

- Demonstrated that systemically stable expression of OPG using rAAV is capable of decreasing osteolytic bone damage in a mouse model of metastatic breast cancer.
- Established that doxorubicin acts independently of the influence of OPG on TRAIL binding.

Year 3:

- The OPG treatment although effectively restores bone, fails to prevent the growth of tumor cells in the bone.

Year 4:

- Combination therapy of OPG with bisphosphonate indicate significant benefit in reducing the growth of MDA-MB-435 cells in a mouse model of breast cancer dissemination.

REPORTABLE OUTCOMES

(Papers published or communicated)

- Isayeva, T., Ren, C., and Ponnazhagan, S. Intraperitoneal transduction of adeno-associated virus 2 expressing angiostatin and endostatin synergistically augments paclitaxel therapy and tumor-free survival in a mouse model of epithelial ovarian cancer. Gene. Ther. 2006 ().

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CONCLUSIONS

We produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. We have successfully determined that sustained expression of OPG using recombinant AAV greatly reduces osteolytic bone damage in a mouse model of bone metastatic breast cancer. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of $\alpha 4\beta 1$ integrin. We have characterized the nature of the treatment effects of OPG both on bone and on the cancer cells. As well, we determined the toxicity-related issues of this treatment. We observed significant inhibition of tumor growth in mice treated with the bisphosphonate pamidronate in combination with rAAV-OPG.Fc compared to pamidronate alone and untreated animals suggesting the potential of combination therapy for breast cancer.

PERSONNEL RECEIVING PAY FROM THIS GRANT

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REFERENCES

N/A

APPENDICES

N/A